

Biodegradation of Isophthalonitrile by Sporosarcina NKCT1101 Isolated from Wastewater of the Washing Workshop

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Abstract: Four strains NKCT1101, NKCT1102, NKCT1103 and NKCT1104 isolated from wastewater of the washing workshop can utilize Isophthalonitrile (IPN) as the sole source of carbon and nitrogen. From the result of the physiological and biochemical experiments, the bacteria were similar to *Sporosarcina*, *Micrococcus*, *Staphylococcus* and *Azotobacter* respectively. In the study, several batch experiments were conducted to investigate the different degradation rate of the four strains, the biodegradation of IPN by NKCT1101. The result showed that degradation rate of IPN by NKCT1101 (about 90%) was much higher than that by NKCT1102, NKCT1103 and NKCT1104 (all about 70%). NKCT1101 could degrade IPN observably at the low biomasses. Even of different initial concentration, NKCT1101 could degrade IPN to a low residual concentration (10mg l^{-1}). The additional carbon glucose and the additional nitrogen urea and Ammonium sulfate which not affect the degradation rate of NKCT1101. Finally NKCT1101 transformed IPN to corresponding acid by nitrilase. And the molecular weight of the nitrilase was 76kD.

1. Introduction

As one of the nitriles, Isophthalonitrile (IPN) is also a toxic organo-cyanide compound (CY.Chen, 2010) which is an important organic intermediate and is widely used in synthesis of chlorothalonil and is the ingredient of PVC, Phenyl dimethylamine and Polyurethane. The increased distribution of IPN in the environment predestines them as potential pollutants of soils and industrial waste waters and requires their detoxification. In 1969, IPN was started to be produced by America and Japan. In the 1980s, the IPN was begun to be widely produced and used in China which exhibited harmful effects on humans and the environment.

The pathway of nitriles' degradation includes chemical and biological. Organic nitriles may be synthesized by a variety of reactions (Crosby et al., 1994) and can be in turn converted into acids, amides, amines and other compounds, having been applied in many organic syntheses (Ludmila Martínková et al. 2009). Ag nanoparticles can efficiently catalyze hydration of nitriles to amide in water under neutral conditions (A. Y. Kim et al. 2011). The hydrolysis of valeronitrile in pure supercritical water can provide technically significant results without the use of catalysts (Michael

Sarlea et al. 2010). Under the basic condition that role of the oxide anion radical in the hydrolysis mechanism, sono-hydrolysis of nitriles to carboxylic is swift and efficient (Pascal Lignier et al. 2011). They are some of the newly chemical methods for the degradation of nitriles all of the above. Furthermore, biological methods are not only cost effective, but also environmental friendly as they lead to innocuous end products. The success of bioremediation mainly depends on the isolation of microbes that use nitriles as a sole source of carbon, nitrogen and energy (Sorokin et al., 2007).

A variety of microorganisms have been described for nitriles' enzymes degradation. A mesophile Gram-negative bacterium, *Acidovorax facilis* 72W, producing a heat-stable nitrilase, has also been isolated from soil (Gavagan et al. 1999). A bacterial strain *Paracoccus* sp. SKG capable of utilizing acetonitrile as a sole source of carbon and nitrogen was isolated from the chemical waste samples. This strain is capable of degrading acetonitrile in the presence of other organic solvents such as methanol, ethanol and dimethylformamide (M. Santoshkumar^a et al. 2011).

In this study, NKCT1101 was isolated from the wastewater of the washing workshop which used IPN as the sole source of carbon and nitrogen, and on the suitable condition, IPN can be effectively degraded. Moreover, the IPN can't process α -oxidation., so we focus on the other three pathways of the biodegradation. Finally, results obtained in this work provide the fundamental studies of biodegradation of IPN.

2. Materials and methods

2.1 Chemicals

Isophthalonitrile (IPN) was produced by Taizhou force chemical Co., LTD, China. Media ingredients were purchased from Chemical, China. All other chemicals used in this study were of analytical grade.

2.2 Culture Mediums and Condition

The ingredients of the enrichment medium were as follows (grams per liter): peptone 10.00; NaCl 8.0; KH_2PO_4 1.0; glucose 1.0. Selective medium (grams per liter): NaCl 1; KH_2PO_4 1.5; KH_2PO_4 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5; IPN (0.02, 0.04, 0.06, 0.08, 0.10, gradually increasing). All the media were sterilized by autoclaving at 121 °C for 15 min. The pH of these media was between 6.8 and 7.2. All cultures were incubated on a rotatory shaker (150 rpm) at 30 °C, for 7d.

2.3 Screening Stains for Degradation of IPN

The sludge samples containing several natural bacteria that could detoxify the IPN were from the washing workshop of Taizhou force chemical Co., LTD, China. Four grams of the sludge samples were added to 50ml enrichment medium, and five samples of different sample location were chosen. Then transferred 5% inoculum to fresh selective medium supplemented with 0.02% IPN as sole carbon and nitrogen source. Every seven days inoculated 5% of the culture into next fresh selective medium with IPN gradually up to 0.1% by 0.02% every time.

Inoculated the last liquid medium on LB agar plates with 0.1% by dilution-plate method, and selected the single colonies with different characters grew out in the LB agar, then transferred the single colonies to the selective medium with 0.1% IPN. Check the IPN degradation of each single colony, finally got IPN-degrading bacteria.

2.4 Analysis of IPN

IPN in liquid culture was determined by UV spectrophotometer at 207nm. Samples were centrifuged (10000r/min, 10min) by High-Speed Refrigerated Centrifuge (Himac CR 21G) and diluted into measuring range before determined.

During the degradation, MS (Finnigan TSQ Quantum ultra AM, USA Thermo) was used to analyse the structural changes. Samples were injected by ultrapure grade water after removing precipitate by centrifuging.

2.5 IPN-degradation Bacteria

Through of a series of physiological and biochemical and morphological observation, got the strain identification. Measuring the absorbance at 600nm monitored the microbial growth.

2.6 Enzyme Analysis

2.6.1 Preparation of Cell-free Extract

Cells were harvested from LB medium containing 0.4% IPN. Harvested cells were washed by sodium phosphate buffer (pH7.0) for twice and centrifuged for 10min at 10000 (4°C), then dissolved in buffer again. The cell suspension were disrupted by Ultrasonic Cell Disruptor (XO-1200D) at a power of 420W for 9min (6s disrupting, 4s interval), and then centrifuged for 10min at 8000g (4°C) to get the liquid supernatant as the extract stored at 4°C before used.

2.6.2 Protein Determination

Protein was determined by Bradford methods.

2.6.3 Enzyme Assay

One unit of the IPN-degrading enzyme was defined as the amount of enzyme producing 1 μ mol ammonia min⁻¹ at pH 7.0, 35°C and 20 mM IPN as a substrate. Ammonia was analysed by the phenol/hypochlorite method (Fawcett and Scott 1960).

2.6.4 Enzyme separation and purification

The crude sample was firstly purified by fraction precipitation by ammonium sulfate. Then the sample (4ml) was added uniformly into the column Sephadex G-100 (2 \times 55cm) and washed by sodium phosphate buffer (pH7.0) with peristaltic pump. UV detector at 280nm was used to record the elution curve. The flow rate was 0.5 ml/min, and 30ml fractions were collected, every 2ml fractions in a sample tube for enzyme assay.

2.6.5 Protein Molecular Weight

The molecular weight of the purified enzyme was detected by polyacrylamide gels electrophoresis (Laemmli, 1970).

3. Results and Discussion

3.1 Isolation and Identification of the Isophthalonitrile (IPN) Strains

Five samples of different sample location were inoculated in enrichment medium and selective medium and finally there were several different colonies grown on the agar plates, just found four colonies could utilize isophthalonitrile (IPN) as the sole source carbon and nitrogen. These four

colonies with different cell morphology and physiological properties were different in degradation rate. The four strains were designated as NKCT1101, NKCT1102, NKCT1103 and NKCT1104 respectively. It can see that the degradation of NKCT1101 was more quickly than NKCT1102, NKCT1103 and NKCT1104, for the degradation of IPN NKCT1101 was obviously faster than NKCT1102, NKCT1103 and NKCT1104 since the second day of the inoculation. The degradation rate of NKCT1101 was about 90%, although the highest degradation rate of NKCT1102, NKCT1103 and NKCT1104 was 66.9% (Fig. 1). At the same time, the strains grew on the agar plate nearly 80% was NKCT1101. So NKCT1101 was the main IPN degradation strain. The cell morphology of the four strains was observed by microscope and they're all small coccoid, but they were different in physiological and biochemical characteristics (Table. 1). For example NKCT1101, NKCT1102 and NKCT1103 were gram positive, NKCT1104 was gram negative and they were also different in physiological properties (Table. 1). As the results of the taxonomic characterization, according to Bergey's Manual of Determinative Bacteriology, NKCT1101 was *Sporosarcina*, NKCT1102 was *Micrococcus*, NKCT1103 was *Staphylococcus* and NKCT1104 was *Azotobacter*.

Table 1 Taxonomic characterization of the bacterium

Criteria	NKCT1101	NKCT1102	NKCT1103	NKCT1104
Cell morphology	Small coccoid	Small coccoid	Small coccoid	Small coccoid
Staining	Gram positive Spore positive	Gram positive	Gram positive	Gram negative
Physiological Properties	Aerobic	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe
MR	-	-	-	-
VP	-	-	+	-
Indole	+	-	-	-
H ₂ S	+	-	+	+
Starch	+	+	+	+
Catalase	+	+	+	+
Citrate utilization	+	-	-	+
Oxidase	+	+	+	+
Sugar fermentation tests	No acid and gas produced from glucose, lactose	No acid produced from glucose, lactose, gas produced from lactose	Acid produced from glucose, not lactose, no gas produced from lactose, glucose	No acid and gas produced from glucose, lactose

Represent the results of the Physiological Properties: “-” negative, “+” positive

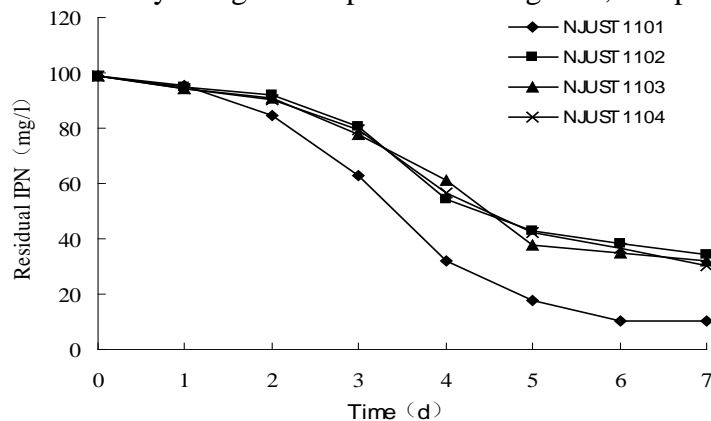


Figure. 1 The IPN degradation of the four strains under the same condition.

3.2 Biodegradation of IPN by NKCT1101

As mentioned above NKCT1101 was the main strain for the IPN-converting, NKCT1101 would be the main research strain in IPN detoxification. NKCT1101 grown in selective medium with about 0.1% IPN showed the fewer biomasses but high detoxification ability. At the fifth day the bacterium was observed at the maximum growth and IPN degraded to a low level (Fig. 2). The solubility of IPN in water is 700mg l^{-1} but within the medium the solubility can't reach 500mg l^{-1} . At different initial concentration 50mg l^{-1} , 100mg l^{-1} , 200mg l^{-1} , 300mg l^{-1} , 400mg l^{-1} , NKCT1101 could also degrade IPN to a low level about 10mg l^{-1} (Fig. 3). So at the initial concentration 400mg l^{-1} , the degradation rate of IPN was up to 99%. The additional carbon glucose and the additional nitrogen urea and Ammonium sulfate which slowed down the degradation efficiency but not affect the degradation rate of NKCT1101.

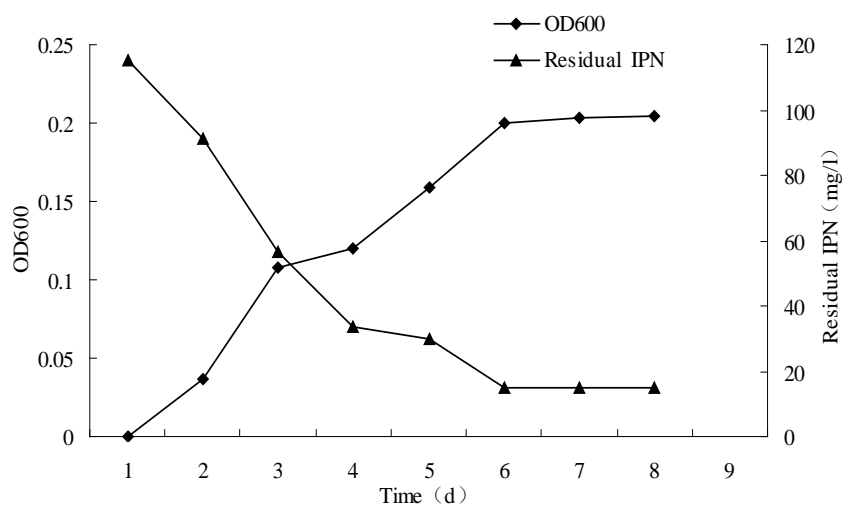


Figure. 2 Growth and utilization of IPN by NKCT1101.

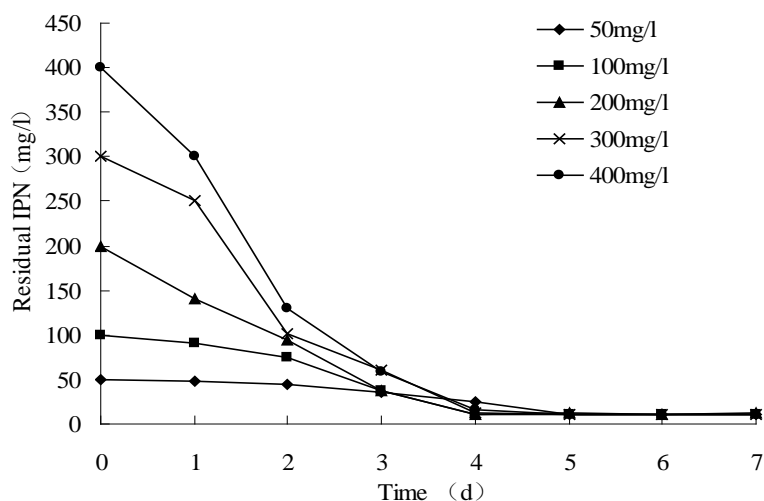


Figure. 3 Degradation of IPN at different initial concentration by NKCT1101

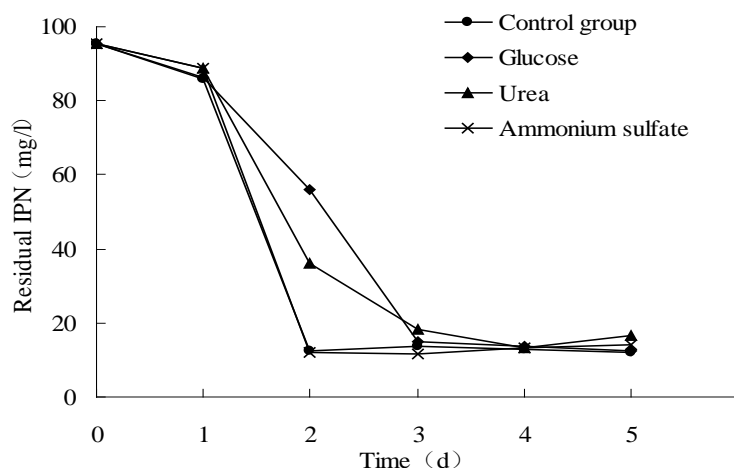


Figure. 4 The effect of biodegradation of IPN by additional carbon glucose, the additional nitrogen urea and Ammonium sulfate

The degradation products of IPN may be different due to the enzyme secreted from the microorganism and MS was used to detect the products. The MS profile (Fig. 5) below showed the characteristic peak of isophthalic acid which was detected in the biodegradation products of NKCT1101, and it's only found isophthalic acid but no m-xylene-diamine and m-xylene. In addition, m-xylene-diamine was not degraded when used as a substrate at the same condition. So it's demonstrated that nitrilase was produced by NKCT1101 during the biodegradation of IPN.

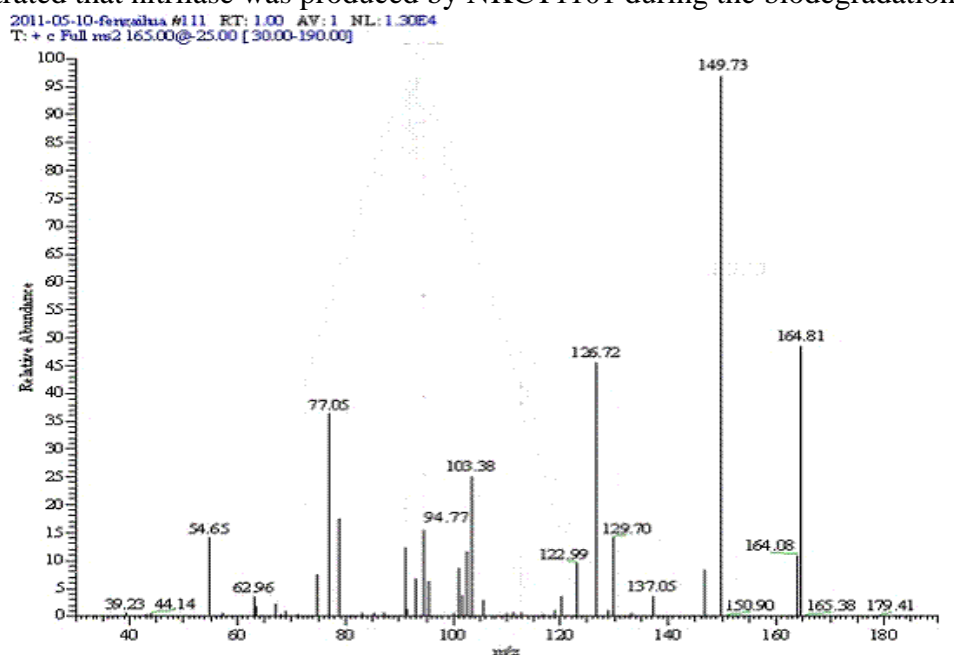


Figure. 5 The analysis of the biodegradation products of NKCT1101 by MS

3.3 Enzyme Analysis

Cell-free extracts was prepared by ultrasonication, leaving the liquid supernatant after centrifugation. The crude cell-free samples were purified by fraction precipitation by ammonium

sulfate and column (2×55cm) Sephadex G-100, finally got the enzyme band by polyacrylamide gels electrophoresis. According to the mark, the molecular weight of the nitrilase was 76 kD.

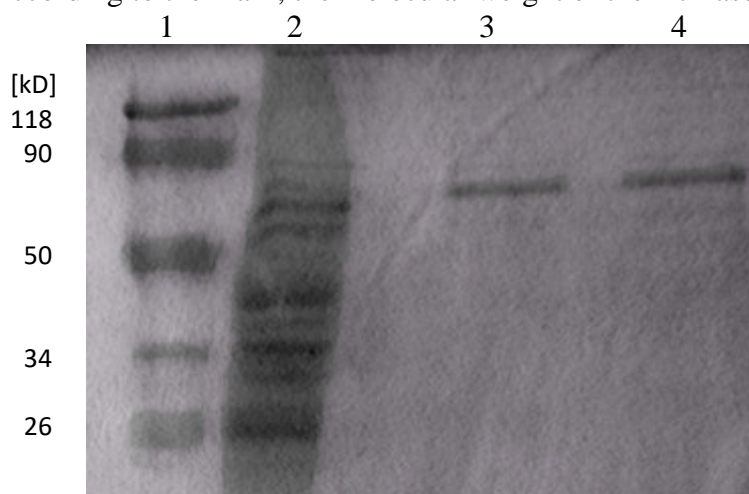


Figure. 6 Analysis of nitrilase by sodium dodecyl polyacrylamide gels electrophoresis. Lanes: 1 Molecular mass standard proteins, 2 crude extract, 3 sample from Sephadex G-100 column the second time, 4 sample from Sephadex G-100 column the first time

4. Conclusion

Four strains NKCT1101, NKCT1102, NKCT1103, NKCT1104 were screened from the wastewater of the washing workshop could use Isophthalonitrile (IPN) as the sole carbon and nitrogen source. The four strains were of different genus and degradation rate but they were all coccoid. Although lots of bacillus were described could detoxicate nitriles, like *Alcaligenes faecalis* (Yamamoto, 1991), *Thermophile Bacillus* sp. (Ludmila Kabaivanova, 2008), *Klebsiella pneumoniae* ssp. ozaenae (Stalker, 1988). *Rhodococcus* and *Pseudomonas* were also main genus in degrading nitriles (C. O'Reilly, 2003). NKCT1101 had a higher degradation rate than NKCT1102, NKCT1103, NKCT1104 and 90% of the bacterium after screening was NKCT1101. Hence NKCT1101 took the important part in degrading IPN and studying the biodegradation of IPN. Biomasses of NKCT1101 grown in selective medium were low but the corresponding biodegradation rate of IPN was high. It's indicated that the ability of detoxication IPN for NKCT1101 was much higher. Although there were four different way of biodegrading IPN, it's finally confirmed the IPN was transformed to corresponding acid by nitrilase. After separation and purification, the nitrilase produced by NKCT1101 was referred to Protein Marker and the molecular weight was 76 kD.

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